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The figure consists of two circular diagrams representing the structure of a protein complex. The left diagram shows the native state with subunits Q, A, L, and K. The right diagram shows the denatured state with subunits A, Q, O, and K. Both diagrams include labels for specific residues and bonds.

BASE-p1

Pairs of synthetic peptides designed in such a manner that they pair specifically with one another to form a heterodimer and then, once paired, preferentially fold as a helical heterodimer. The peptide members of the pair may be of any length, provided they are sufficiently long that they are stable in the heterodimeric form and are able to fold into a helical configuration. The synthetic peptide pairs of the present invention can be used as affinity reagents to isolate associated molecules. In addition, because the heterodimer is preferentially formed and very stable under physiological conditions, the synthetic peptides of the present invention are suitable for use *in vivo*. The synthetic peptide pairs can be used, for example, for *in vivo* applications in which two molecules or components necessary for a given event must be brought together for the event to occur. The synthetic peptide pairs of the present invention make it possible to bring the components together with great specificity and affinity. Alternatively, the synthetic peptide pairs can be designed to prevent binding or interaction of molecules (e.g., DNA and its DNA binding protein) necessary for an event to occur. The synthetic peptide pairs can also be used as a biodegradable molecular velcro, such as in grafting and for artificial sutures.

AMINO ACID SEQUENCES WHICH PAIR SPECIFICALLY

Description

Background

5 Specificity in protein-protein interactions is not well understood, although considerable effort has been expended in trying to explain what determines such interactions. It would be very useful to know what characteristics of an amino acid sequence contribute to protein-
10 protein interactions.

Summary of the Invention

 The present invention relates to pairs of synthetic peptides designed in such a manner that they pair specifically with one another to form a heterodimer and then,
15 once paired, preferentially fold as a helical heterodimer. The present invention further relates to a method of making pairs of synthetic peptides which bind preferentially to one another and to methods of producing such peptide pairs. The peptide members of the pair may be of
20 any length, provided they are sufficiently long that they are stable in the heterodimeric form and are able to fold into a helical configuration. The two members of a pair will typically be of the same length, although this is not required. In general, the individual peptides will be at
25 least 6 to 8 amino acid residues in length, generally at least 12-14 amino acid residues in length and will preferably be at least 16-20 amino acid residues in length. In one embodiment, the individual peptides will be at least 20-23 amino acids in length. In another embodiment, the
30 individual peptides will be 24-30 amino acids in length, particularly to 30 amino acids in length. There is no

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upper limit on individual peptide length. More than one peptide "repeat" or unit may be combined in a peptide pair of the present invention, if desired. That is, more than one peptide which pairs specifically with another peptide to form a heterodimer and, once paired, forms a coiled-coil helical heterodimer can be present in a synthetic peptide of the present invention; the second member of the peptide pair also includes multiple peptide repeats or units, whose amino acid sequences are designed to pair specifically and avidly with the first peptide repeats and preferentially form a coiled-coil helical heterodimer. In particular, it relates to pairs of synthetic peptides (whose members are designated ACID-p1 and BASE-p1 herein and A-1 and B-1 in U.S. Application, Serial No. 07/829,140, to which the subject application claims priority) in which the amino acid sequence has been designed in such a manner that the two members of the pair "stick" to each other with great specificity and avidity. In contrast, neither peptide sticks efficiently to itself. The amino acid residues which comprise the members of the peptide pairs can be naturally-occurring amino acid residues, non-naturally occurring amino acid residues or modified amino acid residues. As described herein, under physiological conditions of temperature and pH, homodimers of the synthetic peptides are very unstable, relative to heterodimers of the synthetic peptides and, in an equilibrium mixture of the two peptides, the heterodimer is favored over the homodimers by at least 1-million-fold.

In one embodiment of the present invention, two peptides (designated A-1 and B-1 in U.S. Application, Serial No. 07/829,140) designed to bind one another specifically and avidly and to preferentially form a helical heterodimer have been synthesized and characterized, using known methods. As described herein, under physiological conditions, individual peptides have been shown, using

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circular dichroism (CD) spectroscopy, to be predominantly unfolded in isolation and when combined, to associate preferentially to produce stable, parallel, coiled-coil (helical) heterodimers. Further, the degree of preference
5 for the heterodimers has been estimated by studying the stability of the heterodimers and homodimers, using CD methods; results showed that the heterodimer has much greater stability than either of the homodimers. The observed difference in stability between the homodimers
10 and the heterodimer suggests that the heterodimer is preferred over the homodimers by at least 1-million-fold. In addition, the oligomerization state, helical content and helix orientation can be assessed, using, respectively, sedimentation equilibrium studies, CD and disulfide
15 bonding of the peptides in the desired parallel orientation, followed by measurement of the concentration dependence of stability.

In a specific embodiment of the present invention, two peptides, each containing two and 14 amino acid re-
20 peats designated, respectively, ACID-p1 and BASE-p1, (designated A-1 and B-1 in a related U.S. Application, Serial No. 07/829,140) were designed to bind to one another specifically and avidly and to preferentially form a helical heterodimer. The two peptides have been syn-
25 thesized and characterized using known methods. Using the same methods as described above and further described herein, under physiological conditions, the individual peptides (ACID-p1 and BASE-p1) were shown to be pre- dominantly unfolded and an equimolar mixture of ACID-p1
30 and BASE-p1 has been shown to preferentially form heterodimers which are stable, parallel in orientation and highly helical. The heterodimer has been shown to have greater stability than either of the homodimers. The amino acid composition and order of the constituent pep-
35 tides were designed to introduce destabilizing electro-

static interactions in the homodimers that would be relieved in the heterodimers. The two peptides differ only at two positions (designated e and g, as defined below) by a single amino acid. The ACID-pl peptide contains, at these two positions, an acidic amino acid, such as glutamic acid; the BASE-pl peptide contains, at these two positions, a basic amino acid, such as lysine. Measurements of the specificity of dimer formation demonstrate that these peptides have at least 10^5 -fold preference for the heterodimeric state. Studies of the pH and ionic strength dependence of stability confirm that electrostatic destabilization of the homodimers provides the primary driving force for the specificity of heterodimer formation. Finally, the oligomerization state, helical content and helix orientation were assessed using respectively, sedimentation equilibrium studies, CD and disulfide bonding of the peptides in the desired parallel orientation, followed by measurement of the concentration dependence stability.

The synthetic peptide pairs of the present invention can be used as affinity reagents to isolate associated molecules. For example, a synthetic peptide pair, such as ACID-pl and BASE-pl, can be used in place of biotin-avidin (e.g., in a biotin/streptavidin affinity method), epitope tagging or immunoaffinity purification methodology. In addition, because the heterodimer is preferentially formed and very stable under physiological conditions, the synthetic peptides of the present invention are suitable for use in vivo. In this embodiment, for example, one member of the synthetic peptide pair (e.g., ACID-pl) can be produced as part of a chimeric or hybrid peptide product (chimeric peptide 1), which includes the synthetic peptide (one member of the peptide pair) and an additional component which is not a synthetic peptide of the present invention. The second component can be a peptide, poly-

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peptide, glyco- or other protein, a detectable label or a small organic molecule, which is to be joined or brought into contact with another molecule. The other member of the synthetic peptide pair can be, for example, included

5 in a second chimeric or hybrid product (chimeric peptide 2) which includes the synthetic peptide (e.g., BASE-pl) and the molecule with which the additional component of the first chimeric product (chimeric peptide 1) is to bind or otherwise interact with. Such chimeric or fusion

10 peptides are also the subject of this invention. They can be produced using known techniques, such as recombinant production methods or chemical (synthetic) methods. They can be produced as one product (e.g., a chimeric peptide which includes both components as produced) or the com-

15 ponents can be produced individually and then joined, using known methods. The synthetic peptide pairs can be used, for example, for *in vivo* applications in which two molecules or components necessary for a given event must be brought together for the event to occur. The synthetic

20 peptide pairs of the present invention make it possible to bring the components together with great specificity and affinity. Alternatively, the synthetic peptide pairs can be designed to prevent binding or interaction of molecules (e.g., DNA and its DNA binding protein) necessary for an

25 event to occur. The synthetic peptide pairs can also be used for biodegradable procedures involving grafting and artificial sutures.

Brief Description of the Drawings

Figure 1 is a coiled-coil helical wheel representation of amino acid residues present in peptide A and

30 peptide B at the locations indicated.

Figure 2 is a coiled-coil wheel representation of the sequence of the ACID-pl/ACID-pl peptide homodimer (Panel A), the BASE-pl/BASE-pl peptide homodimer (Panel B) and

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the ACID-pl/BASE-pl peptide heterodimer of the present invention (Panel C). In this representation, the sequence of the peptide ACID-pl and peptide BASE-pl is arrayed on a coiled-coil diagram in which the helices of the dimer are viewed from the N-terminus (with the helix axis projecting into the page). The sequence of the coiled-coil peptide is divided into positions of the heptad repeat, labeled a-g; amino acid residues at positions a and d make up the 4-3 hydrophobic repeat characteristic of coiled coils.

10 Amino acids are represented by their respective one-letter code.

Figure 3 presents circular dichroism spectra of the ACID-pl peptide, the BASE-pl peptide and the ACID-pl/BASE-pl heterodimer at 37°C in phosphate buffered saline (PBS), pH 7.0 (physiological conditions).

Figure 4 presents a circular dichroism melting curve of the ACID-pl/BASE-pl heterodimer at a wavelength of 222nm.

Detailed Description of the Invention

20 The present invention is based on Applicant's discovery of characteristics of pairs of peptides necessary and sufficient for such peptides to bind or pair specifically and avidly to one another, to form heterodimers, and for the resulting heterodimers to preferentially fold as a helical heterodimer. As a result of this discovery, Applicant has designed and produced peptide pair members which, when mixed, associate preferentially to form a stable, parallel coiled-coil heterodimer, rather than their respective homodimeric forms. The peptides of the present invention may be of any length, provided that they are sufficiently long to be stable when paired with the other member of the peptide pair (i.e., in the heterodimeric form) and to be able to allow the heterodimer to fold into a helical configuration. Generally, the pep-

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tides will be at least 6 to 8 amino acid residues, generally at least 12 to 14 amino acid residues in length and preferably, at least 16 to 20 amino acid residues in length or between 20 or 30 amino acid residues long. In one embodiment, each member of the peptide pair has two 14 amino acid residue "repeats" (two 14 amino acid units) resulting in each pair member being 28 amino acids long. In another embodiment, each pair member is 30 amino acids long. In another embodiment, each pair member is 100 amino acids long. There is no upper limit on the length of peptide members and the appropriate length will be determined by such considerations as the context in which a peptide pair is to be used (e.g., in vitro or in vivo, isolated method, therapeutic or diagnostic techniques).

More than one peptide repeats or units of the present invention can be combined (i.e., present in a single multi-unit peptide) if desired. For example, more than one repeat peptide designed to pair specifically and avidly to a second peptide, as described herein (e.g., designated peptide ACID-p1 herein and A-1 in U.S. Application Serial No. 07/829,140, to which the subject application claims priority), can be combined in a first multi-unit peptide and more than one repeat of the second peptide, (e.g., designated peptide BASE-p1 herein and B-1 in U.S. Application Serial No. 07/829,140) also described herein, can be combined in a second multi-unit peptide and the two multi-unit peptides used as the two members of the peptide pair. The individual peptide units in the multi-unit peptide can be the same or different. In one embodiment described herein, the individual peptide units differ within a peptide (e.g., there are two or more peptide units which are not identical in sequence) but are the same between peptides (e.g., the two members of the peptide pair each contain the same peptide unit(s) as the other member). Amino acids in the peptides can be natu-

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rally-occurring amino acids, non-naturally-occurring amino acids or modified amino acids. The members of a peptide pair stick avidly to the other member of the pair, but do not bind efficiently to a like member of the pair. The

5 following is a description of the rationale underlying the design of the peptide pairs; design synthesis and characteristics of peptide pairs, particularly of peptides ACID-p1 and BASE-p1, of the present invention; and uses therefor.

10 Figures 1 and 2 are coiled-coil helical wheel representations of 30 amino acid residues of the ACID-p1/ACID-p1 peptide homodimer (Figure 2, Panel A), the BASE-P1/BASE-P1 peptide homodimer (Figure 2, Panel B) and the ACID-p1/BASE-p1 peptide heterodimer (Figure 2, Panel C).

15 The sequence of the coiled-coil peptide represented is divided into positions of the heptad repeat, which are labeled a through g. Selection of the appropriate type of amino acid residue for each of the positions relied on studies of naturally-occurring leucine zipper peptides, particularly the Fos/Jun leucine zipper heterodimer and the GCN4 leucine zipper homodimer, as described below.

20 The peptide pairs described herein were designed based on principles learned from Applicant's study of coiled-coil amino acid sequences, including the heterodimeric Fos/Jun leucine zipper. The peptide pairs are designed in such a manner that, in isolation, the individual peptides are unfolded and do not join or stick to a like peptide efficiently (i.e., two of the same peptide pair members do not stick efficiently together to form homodimers). In contrast, when mixed together, the peptide pair members join preferentially to form a stable heterodimeric coiled-coil. The primary driving force for the specificity of binding to form a heterodimer is electrostatic destabilization of the homodimers. As shown by

25 30 35 results presented herein, the designed peptides fold as

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parallel, helical dimers which have a great (at least about 10^5 -fold) preference for the heterodimeric state.

Previous work had demonstrated that an approximately 35 amino acid leucine zipper region from Fos and from Jun is necessary for preferential heterodimer formation. Landschulz, W.H. *et al.*, *Science*, 240:1759-1764 (1988); Ransone, L.J. and I.M. Verma, *Ann. Rev. Cell Biol.* 6:539-557 (1990). Studies of the Fos and Jun leucine zipper heterodimer, as described in Example 1, showed that eight residues at positions e and g of the coiled-coil peptide (heptad repeat) are the primary determinants of specificity of pairing of the two peptides (Fos and Jun) to form a heterodimer. As described in Example 1, 8 residues from Fos and from Jun, in the background of the GCN4 leucine zipper, have been shown to be sufficient to mediate preferential heterodimer formation. Amino acid residues at all other positions of the heptad can be substituted without significant loss of specificity of Fos/Jun pairing. Residues present at positions e and g in the Fos peptide are predominantly acidic. There are many basic residues at positions e and g in the Jun peptide. Studies of the pH dependence of stability suggest that, at neutral pH, Fos is destabilized by these acidic residues and Jun is slightly destabilized by basic residues. Under the same conditions of pH, the Fos/Jun heterodimer has greater stability, due to relief of destabilizing interhelical electrostatic interactions and potential ion pairs.

Examination of the x-ray structure of the GCN4 leucine zipper shows that amino acid residues at positions e and g' (and g and e') are close in space. The residues present at these positions are of opposite charge in the GCN4 homodimer and are seen to participate in interhelical ion pairs. O'Shea, E.K. *et al.*, *Science*, 254:539-544 (1991).

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Relying on the above-described information about the Fos/Jun leucine zipper and the GCN4 leucine zipper, pairs of peptides which pair specifically and avidly to one another and subsequently preferentially form a helical heterodimer have been designed and these peptides have been synthesized and characterized.

In the peptide pairs of the present invention, charged amino acid residues are present at positions e and g (and e' and g'; Figure 1). In one peptide (in Figure 1, designated peptide ACID-pl), a negatively charged amino acid, such as glutamate (glutamic acid), is present and in the second peptide (in Figure 1, designated peptide BASE-pl), a positively charged amino acid, such as lysine, is present. As shown in Figure 1, a negatively charged amino acid residue is present at e (amino acid residues 4, 11, 18, 25) and g (amino acid residues 6, 13, 20, 27) in peptide ACID-pl and a positively charged amino acid is present at e' (amino acid residues 4, 11, 21, 25) and g' (amino acid residues 6, 13, 20, 27) in peptide BASE-pl. A hydrophobic amino acid, such as leucine, is present at positions a and d (peptide ACID-pl) and a' and d' (peptide BASE-pl). Leucine is a preferred choice because it is the most common amino acid at these positions in naturally-occurring coiled coils. However, not all amino acid residues at these positions must be hydrophobic; there may be other (non-hydrophobic) amino acids present at these positions, provided that those present at these positions in aggregate are predominantly hydrophobic. In determining appropriate residues for these positions, reference can be made to work by Hodges and co-workers insert (Hodges, R.S. et al., J. Biol. Chem., 256:1214-1224 (1981); Hodges, R.S. et al., Peptide Res., 1:19-30 (1988); Lau, S.Y. et al., J. Biol. Chem., 259:13253-13261 (1984)) and Conway and Parry (Int. J. Biol. Macromol., 12:328-334 (1990) and Example 1. In one embodiment of the present

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invention, an asparagine is present at the second a position (in peptide ACID-p1, position a and in peptide BASE-p1, position a', both of which represent amino acid 14) in order to favor the parallel orientation and discourage
5 higher order oligomerization. Asparagine is present at the corresponding position in the GCN4 leucine zipper, which is a peptide which folds as a two-stranded parallel coiled coil. The amino acid residues present at positions b, c and f, as represented in Figure 1 can be very varied;
10 almost any combination of amino acid residues can be used, provided that there is an appropriate distribution of hydrophilic amino acid residues at these positions. Selection of amino acid residues appropriate for inclusion at positions b, c and f can be made with reference to
15 Applicants' work (e.g., Example 1) and work by Conway and Parry (see above).

In one embodiment of the present invention, as represented in Figure 2, small, uncharged amino acid residues, particularly those which are good helix formers, are
20 present at positions b and c and b' and c'. Such small, uncharged amino acid residues as alanine and glutamine are used at these locations in order to prevent residues at b and c and b' and c' from interacting with residues at positions e and g and e' and g' and, as a result, competing with the desired interhelical interactions. In
25 addition, polar residues included in the peptide pair members increase solubility of the peptide. In this embodiment, charged residues, such as glutamate and lysine, are included at position f (and f', see Figure 1)
30 because many charged residues are found at this location in naturally-occurring coiled coils. These residues also serve to increase solubility and discourage higher-order oligomerization. A single tryptophan at position f and f' has been included in peptides ACID-p1 and BASE-P1 as a
35 means of facilitating concentration determination by

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absorbance. However, they will generally be a charged residue, such as glutamate or lysine.

Thus, the two members (peptide ACID-pl and peptide BASE-pl) of the synthetic peptide pairs of the present invention have been a general formula which can be described with reference to the coiled-coil helical wheel representation shown in Figure 1. The following amino acid residues are present:

In a peptide A

1. at positions e and g, negatively charged amino acids residues, such as glutamate (glutamic acid);
2. at positions a and d, a hydrophobic amino acid residue, such as leucine; and
3. at positions b, c and f, almost any amino acid residues, provided that there is an appropriate distribution of hydrophilic amino acids, such as a negatively charged amino acid (e.g., alanine, glutamine) at b and c and such as a positively charged amino residue (e.g., glutamate, lysine) at f.

In peptide B

1. at positions e' and g', positively charged amino acid residues, such as lysine;
2. at positions a' and d', a hydrophobic amino acid residue, such as leucine; and
3. at positions b', c' and f', almost any amino acid residues, provided that there is an appropriate distribution of hydrophilic amino acids, such as a negatively charged amino acid residue (e.g., alanine, glutamine) at b' and c' and such as a positively charged amino acid residue (e.g., glutamate, lysine) at f'. In general, proline will not be used in these peptides.

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The amino acid residues present at each of these positions in one embodiment of the two pair members are shown in Figure 2 (by their one-letter code) and in Table 1 (peptide ACID-p1, peptide BASE-p1, respectively, by
5 their three-letter codes).

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TABLE 1AMINO ACID SEQUENCE OF PEPTIDE ACID-p1 AND BASE-p1

Peptide ACID-p1		Peptide BASE-p1	
	1 Ala (A) *		1 Ala (A)
5	2 Gln (Q)		2 Gln (Q)
	3 Leu (L)		3 Leu (L)
	4 Glu (E)		4 Lys (K)
	5 Lys (K)		5 Lys (K)
	6 Glu (E)		6 Lys (K)
10	7 Leu (L)		7 Leu (L)
	8 Gln (Q)		8 Gln (Q)
	9 Ala (A)		9 Ala (A)
	10 Leu (L)		10 Leu (L)
	11 Glu (E)		11 Lys (K)
15	12 Lys (K)		12 Lys (K)
	13 Glu (E)		13 Lys (K)
	14 Asn (N)		14 Asn (N)
	15 Ala (A)		15 Ala (A)
	16 Gln (Q)		16 Gln (Q)
20	17 Leu (L)		17 Leu (L)
	18 Glu (E)		18 Lys (K)
	19 Trp (W)		19 Trp (W)
	20 Glu (E)		20 Lys (K)
	21 Leu (L)		21 Leu (L)
25	22 Gln (Q)		22 Gln (Q)
	23 Ala (A)		23 Ala (A)
	24 Leu (L)		24 Leu (L)
	25 Glu (E)		25 Lys (K)
	26 Lys (K)		26 Lys (K)
30	27 Glu (E)		27 Lys (K)
	28 Leu (L)		28 Leu (L)
	29 Ala (A)		29 Ala (A)
	30 Gln (Q)		30 Gln (Q)

*One letter amino acid symbol.

35 Figure 2, the amino acid residues present at positions a through g of peptide ACID-1 in the heterodimer (Panel C) correspond to amino acid residues 1 through 30 for peptide ACID-p1 in Table 1 and as shown in Table 2.

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TABLE 2

Amino Acid residue		Position in Figure 2	Position in Table 1
5	Leu	a	7
	Asn		14
	Leu		21
	Leu		28
10	Ala	b	1
	Gln		8
	Ala		15
	Gln		22
15	Ala	c	29
	Gln		2
	Ala		9
	Gln		16
20	Leu	d	23
	Leu		30
	Leu		3
	Leu		10
25	Glu	e	17
	Glu		24
	Glu		4
	Glu		11
30	Lys	f	18
	Trp		25
	Lys		5
	Lys		12
35	Glu	g	19
	Glu		26
	Glu		6
	Glu		13
			20
			27

In Figure 2, the amino acid residues present at positions a' through g' of peptide BASE-p1 in the heterodimer (Panel C) correspond to amino acid residues 1 through 30 for peptide BASE-p1 in Table 1 and as shown in Table 2.

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TABLE 3

Amino Acid residue		Position in Figure 2	Position in Table 1
5	Leu	a'	7
	Asn		14
	Leu		21
	Leu		28
10	Ala	b'	1
	Gln		8
	Ala		15
	Gln		22
15	Ala	c'	29
	Gln		2
	Ala		9
	Gln		16
20	Ala	d'	23
	Gln		30
	Leu		3
	Leu		10
25	Leu	e'	17
	Leu		24
	Lys		4
	Lys		11
30	Lys	f'	18
	Lys		25
	Glu		5
	Lys		12
35	Leu	g'	19
	Glu		26
	Lys		6
	Lys		13
	Lys		20
	Lys		27

The amino acid residues can additionally include Cys-Gly-Gly, which is generally added at the N-terminal of the peptide and not part of the 30 amino acid residue peptide of the present invention but, rather, is included for assay purposes only. For example, Cys-Gly-Gly can be added to the peptide (before amino acid residue 1) to

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assist in the assessment of helix orientation (parallel vs. antiparallel) of the heterodimer. To do this, a disulfide-bonded peptide (disulfide-bonded in parallel orientation) is assessed by measuring its stability as a function of peptide concentration; alternatively, molecular weight can be measured as a function of peptide concentration by sedimentation. If the helices are parallel, the disulfide-bonded peptide is expected to have stability independent of peptide concentration and molecular weight equal to the molecular weight of the dimer independent of peptide concentration.

Other short peptide pairs which pair specifically and avidly and subsequently preferentially form helical heterodimers can be produced, using the teachings of the subject application as to the appropriate amino acid residues and known synthetic methods. Once a peptide pair has been produced, its ability to pair specifically and avidly and to preferentially form helical heterodimers can be assessed, as described herein for peptide ACID-p1 and peptide BASE-p1, using known methods.

The members of the peptide pairs of the present invention can be produced using known methods, such as chemical synthesis or recombinant/genetic engineering technology. For example, they can be synthesized as described in Example 2 or in much the same manner as described in Example 1 for the synthesis of the Fos and Jun leucine zipper peptides. Alternatively, the peptides can be produced in an appropriate host cell by expressing DNA or RNA encoding the peptide sequence. As used herein, the term synthetic refers to peptides of the present invention made by any method (e.g., chemically or by recombinant or genetic engineering methods). Peptide pairs of the present invention have many uses, in both in vitro and in vivo contexts. For example, peptide pairs can be used as affinity reagents, in much the same way as

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or as a replacement for other binding pairs. For example, they can be used in place of biotin-streptavidin, epitope tagging methods or immunoseparation methods. In this use, a member of a peptide pair can be attached or linked to
5 another molecule (e.g., another peptide, polypeptide, glyco- or other protein, or a detectable label or small organic molecule) or to a solid support (e.g., a column, particle, filter, plastic plate) by known methods, such as a component of a fusion protein or through a linker (e.g.,
10 the Cys/Gly/Gly referred to above) for attachment to a solid surface. A molecule to which the second member of the peptide pair is attached (e.g., by chemical or recombinant methods) can be separated or isolated by contacting a mixture containing the molecule-peptide pair with the
15 solid surface bearing the second member of the peptide pair, under conditions appropriate for sticking or pairing of the peptide pair members. The fraction of the mixture which is not the molecule to be separated or isolated will not become affixed to the solid support and can be removed
20 simply by separating the solid support from the remainder of the mixture. The bound molecule (bound as a result of pairing of the peptide pair members) can be released from the solid support by, for example, changing the pH and/or temperature of the bound fraction. Such a method of
25 separating or isolating a molecule in this manner can be used, for example, for purification of a molecule to be used for other purposes (e.g., where presence of a particular substance is indicative of the presence or absence of a disease or condition). Peptide pairs of the present
30 method can also be use for in vivo purposes, such as to block, induce or enhance an event in cells (e.g., to interfere with binding of two components in a cell where binding is necessary, thus inducing or enhancing the event). In this embodiment, peptides of the present
35 invention can be produced in cells in which they are to

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act by, for example, expression from a vector, such as a retroviral vector(s) containing DNA or RNA encoding a chimeric peptide or peptides. The chimeric peptide includes the amino acid residues of the peptide of the present invention and, if desired, a peptide or a polypeptide which is not a synthetic peptide of the present invention, such as a peptide which is to act in the cell. Alternatively, peptides of the present invention can be used for radioimaging or to treat diseases, such as malignancies. In the case of malignancies, one member of a peptide pair is expressed in the malignant cell (e.g., from an appropriate vector). The second member of the peptide pair can be labeled, thereby capable of detection or can be joined with an agent which is capable of detection (e.g., a radioactive molecule or substance, such as ricin, toxic to cells) which binds specifically to the cell expressing the first member of the peptide pair. Such an approach can also be used, for example, in treating hyperthyroidism. One member of the peptide pair can be expressed (e.g., from a retroviral or other vector) in the thyroid and the second member of the peptide pair joined to radioactive iodine can be administered to an individual in need of treatment. Pairing of the two peptide pair members results in delivery of the radioactive iodine to the thyroid; continuous delivery occurs until the peptide pair is degraded by the body or otherwise separated.

Members of peptide pairs can be joined to other peptides or proteins (e.g., peptides or proteins to be delivered) by chemical means or included with/incorporated into another peptide or protein which is made by recombinant DNA methods. In either case, a peptide pair member may be joined or present at an end of the peptide or protein or at any internal site at which the protein can tolerate insertion of the peptide pair member (i.e., any

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site at which the peptide pair member can be present and not interfere with the desired function of the other peptide or protein).

EXAMPLE 1 Assessment of the Mechanism of Specificity in
5 the Fos-Jun Heteroprotein

Peptide Synthesis and Purification

Peptides were synthesized using t-BOC chemistry on an Applied Biosystems model 430A peptide synthesizer with standard reaction cycles modified to include acetic an-
10 hydride capping (for a review see Kent, S.B.H., Annu. Rev. Biochem. 57:956-989 (1988). Peptide Jun N corresponds to residues 286-317 of the c-Jun protein (Bohmann, D. et al., Science 238:1386-1392 (1987); Maki, Y. et al., proc. Natl. Acad. Sci. USA 84:2848-2852 (1987)), and peptide Fos N
15 corresponds to residues 162-193 of the c-Fos protein (Van Beveren, C. et al., Cell 32:1241-1255 (1983); Van Straaten, F. et al., Proc. Natl. Acad. Sci. USA 80:3183-3187 (1983)). Ser-295 of c-Jun and Ser-177 of c-Fos have been replaced with tyrosine to facilitate concentration
20 determination by UV absorbance measurements. Peptide GCN4 N consists of residues 250-281 of the GCN4 protein (Hinnebusch, A.G., Proc. Natl. Acad. Sci. USA 81:6442-6446 (1984)). All peptides have the sequence Cys-Gly-Gly appended to the N-terminus, are acetylated at the N-termi-
25 nus, and are amidated at the C-terminus. Peptides were cleaved by either low/high HF cleavage (Immunodynamics, Inc., San Diego, CA) or by trifluoromethanesulfonic acid cleavage (Kent, S.B.H., Annu. Rev. Biochem. 57:956-989 (1988) and were desalted on a Sephadex G-10 column (Pharm-
30 acia) in 5% acetic acid. Final purification was by reverse-phase high performance liquid chromatography (HPLC) (Waters, Inc. and Applied Biosystems) using a Vydac preparative C18 column (2.2 x 25 cm) at 25°C or 50°C. A

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linear acetonitrile-H₂O gradient with segments of 0.1% to 0.2% buffer B increase per minute was used with a flow rate of 20 ml/min. Buffer A consisted of water with 0.1% trifluoroacetic acid, and buffer B consisted of 90% acetonitrile, 10% water with 0.1% trifluoroacetic acid. The identity of each peptide was confirmed by fast atom bombardment mass spectrometry (M-Scan, Inc., West Chester, PA or Mass Search, Inc., Modesto, CA) and was found to be within 1 dalton of the expected mass.

10 Circular Dichroism Studies

Circular dichroism (CD) studies were performed using a 1 cm or 1 mm cuvette (Helma or Uvonic) on an Aviv CD spectrophotometer (model 60DS or model 62DS) equipped with a thermoelectric controller. The buffer used for all CD experiments except pH titrations was 50 mM NaCl, 20 mM NaPO₄ (pH 7.0). All peptide concentrations were determined by tyrosine absorbance (Edelhoch, H. Biochemistry 6:1948-1954 (1967)) at 275.5 nm in 5.4-6 GuHCl (Schwarz/-Mann Biotech Ultra-Pure grade) using an Aviv UV/VIS spectrophotometer (model 18DS or 14DS). The molar ellipticity at 222 nm, 0°C, was measured for the Fos and Jun N, N_{in}, N_{out}, N_{ad}, N_{ee}, disulfide-bonded homo- and heterodimers and for the GCN4 N homodimer. All values were found to be within the range -27,000 to -33,000 deg cm² dmol⁻¹, indicating that the peptides are > 80% helical. Thermal melting curves were determined by monitoring the CD signal at 222 nm as a function of temperature. The pH dependence of stability was measured in 50 mM NaCl, 10 mM NaPO₄ at various pH values. Because the disulfide-bonded GCN4 N dimer is very stable, 2 M GuHCl (Schwarz/Mann Biotech Ultra-Pure grade) was included in the buffer used for monitoring the pH dependence of stability of this peptide. The same overall shape of the pH dependence curve is obtained in the absence of GuHCl. Reversibility was

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checked for all thermal melts. In general, melting curves obtained above pH 8 are not reversible, presumably because of chemical modification and/or degradation (observed by HPLC). Disulfide-bonded dimers that have irreversible melting curves (<75% of folded signal is recovered upon cooling) at pH 7.0 are marked (asterisk). These peptides have undergone chemical modification and/or degradation during the thermal melt, as judged by subsequent HPLC analysis.

-10 Determination of the T_m

The T_m was determined by curve fitting the thermal denaturation curve to the following equation using a nonlinear least squares-fitting program (Kaleidagraph, Synergy Software):

$$15 \quad \theta = \theta_f (0 \text{ K}) + m_f T + \left[\theta_u (0 \text{ K}) + m_u T = \theta_f (0 \text{ K}) - m_f T \right] \\ \left[e^{-\Delta H/RT + \Delta S/R} / 1 + e^{-\Delta H/RT + \Delta S/R} \right]$$

where T is temperature in K; θ is the CD signal at 222 nm; $\theta_f (0 \text{ K})$ is the value for the CD signal of the folded peptide extrapolated linearly to 0 K; m_f is the slope of the temperature dependence of the CD signal for the folded peptide; m_u is the slope of the temperature dependence of the CD signal for the unfolded peptide; $\theta_u (0 \text{ K})$ is the value for the CD signal of the unfolded peptide extrapolated linearly to 0 K; ΔH is the enthalpy of unfolding at the midpoint of the thermal denaturation curve; and ΔS is the entropy of unfolding at the midpoint of the thermal denaturation curve. The T_m is the temperature at which the fraction unfolded is equal to the fraction folded ($\Delta G = 0$), and it is equal to $\Delta H/\Delta S$. The assumptions that were made in using the equation above are as follows: thermal melting curves are two state, described by an equilibrium between unfolded and folded peptide; and the enthalpy and entropy of unfolding are independent of temperature.

Because some of the Fos peptide homodimers and Fos
N_{in}-GNC4 N_{in} peptide heterodimers are not completely folded
at 0°C, the slope of the folded baseline (m_f) and the
value for the CD signal of the folded baseline extra-
5 polated to 0 K (θ_f (0 K)) for each of these dimers were
determined from a melting curve at pH 2.0 (conditions
where the Fos-containing peptides are more stable).

For each peptide the T_m was also determined by taking
the first derivative of the CD signal (θ) with respect to
10 temperature⁻¹ (temperature in K) and finding the minimum
of this function (Cantor, C.R. and P.R. Schimmel, Bio-
physical Chemistry, W.H. Freeman, New York (1980)). All
reported values of T_m are those determined from curve
fitting. The error in the measurement of T_m (estimated
15 from the width of the $d\theta/d(1/T)$ plot and from repeated
curve fits starting from independent points) is $\pm 2^\circ\text{C}$
except in cases in which $20^\circ\text{C} > T_m > 80^\circ\text{C}$, where the error
is $\pm 5^\circ\text{C}$. The determinations of T_m by $d\theta/d(1/T)$ and by
curve fitting agree to within the estimated errors.
20 Additionally, the T_m for each disulfide-bonded dimer was
measured as a function of peptide concentration (over at
least a 2.5-fold range of peptide concentration in the low
micromolar range, as estimated by the ratio of the CD
signal at low and high concentration) to determine if the
25 dimers were associating to higher order oligomers (O'Shea,
E.K. et al., Science 243:538-542 (1989); O'Shea, E.K., et
al., Science 245:646-648 (1989)). Peptide dimers that
were found to have a T_m that is dependent upon peptide
concentration ($\sim 3^\circ\text{C}$ change in T_m over a 3- to 4-fold
30 concentration range), and the T_m reported is the higher of
the two measurements.

Redox Experiments

The disulfide-bonded heterodimer was incubated in
redox buffer consisting of 100-500 μM reduced glutathione

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(Sigma), 100-5-- μ M oxidized glutathione (Sigma), 50 mM NaCl, 10 mM NaPO_4 (pH 7.4) at -23°C in an anaerobic chamber (Coy Laboratory Products, Inc.). Reactions were equilibrated at a total peptide concentration of ~ 10 -50 μ M for 6-16 hr and quenched under anaerobic conditions with concentrated formic acid to a final concentration of 5% by volume (pH < 2). The reaction products were analyzed by microbore HPLC (Waters, Inc.) using a linear acetonitrile- H_2O gradient with segments of 0.1% to 0.25% increase in buffer B per minute. Analytical Vydac C-18 column (0.46 x 25 cm) was used at a column temperature of 25°C , 40°C or 50°C with a flow rate of 0.2 ml/min. Relative concentrations of the disulfide-bonded hetero- and homodimers were determined by integration of the corresponding peaks (absorbance at 229 nm was monitored). Each redox reaction was determined to be at equilibrium by repeating the reaction using an equimolar mixture of reduced peptides as the starting material. The values for ΔG_{spec} obtained from these two different starting points agreed to within 0.1 kcal/mol.

RESULTS

pH Dependence of Stability in the Fos and Jun Leucine Zipper Peptides

The relative stabilities of the Fos and Jun peptide heterodimer and homodimers (Table 1) indicate that the Fos peptide homodimer is substantially less stable than the Fos Jun heterodimer and the Jun homodimer (O'Shea, E.K. *et al.*, *Science*, 245:646-648 (1989)); the new peptides are shorter, lacking both an N-terminal residue and 7 amino acids following the last leucine of the leucine repeat (see Experimental Procedures). As a first step toward identifying sources of stabilization and destabilization in the new Fos and Jun leucine zipper dimers, the pH

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dependence of the disulfide-bonded peptide pairs was studied.

The thermal stability of the Fos and Jun homodimers is pH dependent in a dramatic way; the T_m (melting temperature determined from the midpoint of the thermal unfolding transition, see Experimental Procedures) of the Fos homodimer increases -40°C from neutral to acidic pH, and the stability of the Jun homodimer increases -20°C from neutral to basic pH. The stability of the heterodimer changes approximately as expected from an average of the pH dependence of the homodimers.

These large effects of pH on stability can be explained in part by examining the sequences of the Fos and Jun leucine zippers. A particularly useful representation of the sequence is the coiled-coil diagram in which the helices of the dimers are viewed from the N-terminus with the helix axis projecting into the page. The sequence of the coiled-coil proteins can be divided up into positions of the heptad repeat, labeled a-g (Hodges, R.S. *et al.*, Cold Spring Harbor Symp. Quant. Biol., 37:299-310 (1972); McLachlan, A.D. and M. Stewart (J. Mol. Biol. 98:293-304 (1975)). Residues at positions a and d comprise the 4-3 hydrophobic repeat characteristic of coiled coils, and residues at positions e and g are predominantly charged amino acids that can be involved in intra- or interhelical electrostatic interactions (Hodges, R.S. *et al.*, Cold Spring Harbor Symp. Quant. Biol., 37:299-310 (1972); McLachlan, A.D. and M. Stewart (J. Mol. Biol. 98:293-304 (1975)).

The Fos leucine zipper is very acidic, with a high concentration of acidic amino acids at positions e, g and b. Because each chain has a large net negative charge at neutral pH (each chain has a net charge of -5), one expects that dimer formation would be disfavored due to general electrostatic repulsion between monomers. Ad-

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ditionally, the alignment of four negatively charged side chains at position g along one face of the helix is expected to be a source of intrahelical destabilization. The large increase in stability of the Fos homodimer upon titration to low pH can, thus, be explained to result from the relief of destabilizing intra- and interhelical electrostatic interactions between acidic residues close to the hydrophobic interface of the dimer.

The Jun leucine zipper has a slight net positive charge at neutral pH (dimer has a net charge of +2 at pH 7); in addition, the concentration of charge in the Jun leucine zipper is more spread out than in Fos. These properties are consistent with the less dramatic increase in stability of the Jun homodimer at high pH. Qualitatively, the pH dependence of stability for the Fos-Jun heterodimer changes as expected from an average of the pH dependences for the homodimers. This result suggests that the Fos-Jun leucine zipper lacks dominant stabilizing electrostatic interactions that are unique to the heterodimer; in such a case, a bellshaped pH dependence curve would be expected. In contrast, the stability of the heterodimer increases at acidic pH values, suggesting that intrahelical repulsion (expected from the Fos sequence) is strong.

Electrostatic effects provide a possible explanation for preferential heterodimer formation in the Fos-Jun system. The data suggest that the peptide homodimers are destabilized at neutral pH by residues of like charge, the fos homodimer by acidic residues, and the Jun homodimer, to a lesser extent, by basic residues. The interhelical component of this electrostatic destabilization is relieved in the heterodimer because the Fos and Jun monomers are of opposite charge.

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The Inside Residues Are Responsible for Specificity and pH-Dependent Stability

To probe the specificity of the Fos-Jun leucine zipper interaction further, structurally based hybrid peptides were made by replacing portions of the Fos and Jun sequences with sequence from GCN4. A peptide corresponding to the leucine zipper region from GCN4 forms very stable homodimers (O'Shea, E.K. *et al.*, *Science* 245:646-648 (1989)). As Fos and Jun are likely to fold as coiled coils, the boundary between the Fos or Jun sequence and the GCN4 sequence was set by dividing the helical wheel diagram into two groups of residues: the "inside" group, consisting of the predominantly charged residues at positions e and g, and the "outside" group, consisting of residues from positions b, c and f.

Two sets of hybrid leucine zipper peptides were constructed. One set of peptides has native sequence (N) from Fos or Jun at the inside positions and outside sequence from GCN4; these peptides are referred to as N_{in} . The other set of peptides contains GCN4 sequence inside and Fos and Jun sequence outside; these peptides are referred to as N_{out} .

The preference for heterodimer formation was quantitated from a redox experiment in which an equimolar mixture of the cysteine-containing Fos and Jun peptides is equilibrated in a redox buffer that facilitates disulfide bond formation. K_{redox} is determined from the ration of disulfide-bonded heterodimer to homodimers. The free energy of specificity for heterodimer formation (ΔG_{spec}) is equal to $-RT \ln K_{redox} + RT \ln 2$. These experiments indicate that there is -2.3 kcal/mol (-100:1) preference for heterodimer in the native peptides.

The N_{in} peptides also form heterodimers preferentially, but with reduced specificity (ΔG_{spec} is -1.2 kcal/mol). The decrease in specificity of the N_{in} peptides appears to

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arise from a decrease in stability of the N_{in} heterodimer; the N_{in} heterodimer is less stable than the native heterodimer but the stabilities of the N_{in} homodimers are the same as the corresponding native homodimers (Table 1). In contrast, the N_{out} peptides show essentially no specificity (ΔG_{dec} is -0.1 kcal/mol). As expected if there is no preference for heterodimer over homodimers, the N_{out} heterodimer has a stability that is intermediate between that of the two N_{out} homodimers (Table 1). Therefore, the inside residues of the Fos and Jun leucine zipper are necessary and sufficient to mediate preferential heterodimer formation.

The N_{in} peptides show pH-dependent stability that closely resembles that of the native Fos and Jun peptides. In contrast, the pH dependence for each of the N_{out} dimers does not resemble that of the corresponding native dimer, but resembles that of the GCN4 leucine zipper peptide. Therefore, residues at the inside positions (positions a, d, e and g) are also largely responsible for the pH-dependent stability observed with the Fos and Jun peptide dimers.

Sequence Requirements for Specificity

To investigate more thoroughly the sequence requirements for specificity, all possible combinations of the previously described seven peptides were made (there are 28 possible combinations, and 21 of these are heterodimers), and the stability of each disulfide-bonded dimer was measured by thermal denaturation. There are large differences in stability; the T_m s range from 17°C - 38°C . The free energy of specificity can be approximated by the difference in T_m between a given heterodimer and the average of the T_m s for the corresponding homodimers ($\Delta T_m = T_m(\text{heterodimer AB}) - 1/2 [T_m(\text{homodimer AA}) + T_m(\text{homodimer BB})]$). For the Fos-Jun peptides, ΔT_m appears to be a

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quantitative measure of specificity, as it is linearly related to the free energy of specificity, ΔG_{spec} , with a proportionality constant of 7.4°C/kcal.

Using ΔT_m as a measure of specificity, the hetero-
5 dimeric peptide pairs can be grouped into three classes: specificity, antispecificity and additive. The specificity classes includes peptides pairs with positive, nonadditive differences in T_m ($\Delta T_m \geq +8^\circ\text{C}$); the antispecificity class contains peptide pairs with negative, nonadditive
10 differences in T_m ($\Delta T_m \leq -8^\circ\text{C}$); and the additive class consists of peptide pairs in which the stability of the heterodimer is intermediate between that of the homodimers ($+8^\circ\text{C} > \Delta T_m > -8^\circ\text{C}$).

All peptide dimers combining Jun sequence inside with
15 Fos sequence inside fall into the specificity class. Although the mechanism of antispecificity is not apparent at this time, all members of the antispecificity class have Fos sequence inside combined with GCN4 sequence inside. The other peptide combinations fall into the
20 additive class, with one exception (the Jun $N_{\text{in-Jun Nout}}$ heterodimer falls into the specificity class, for reasons that are not readily apparent). The most striking result is that specific heterodimer formation is observed with
all peptide pairs containing Fos sequence inside combined
25 with Jun sequence inside, regardless of the sequence at the outside position, reinforcing the previous conclusion that the inside residues (positions a, d, e and g) are the major determinant of peptide pairing.

The inside residues consist of the predominantly
30 hydrophobic positions (a and d) and predominantly charged positions (e and g). GCN4-based hybrid peptides containing native Fos and Jun sequence at the hydrophobic positions ($N_{a,d}$) or charged positions ($N_{e,g}$) were made to evaluate the contribution of these groups of residues to specificity. The $N_{e,g}$ peptides form heterodimers with specific-
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ity (ΔT_m and ΔG_{spec}) and stability (T_m) at least as great as that of the native sequences (Table 1). In contrast, the $N_{a,d}$ peptides are slightly anti-specific.

Thus, 8 residues at positions e and g of Fos and Jun are sufficient to mediate preferential heterodimer formation. Although the residues from Fos and Jun that comprise the hydrophobic interface between the helices (positions a and d) are undoubtedly important for stability (Smeal, T. et al., Genes Dev. 3:2981-2100 (1989); Ransone, L.J. and I.M. Verma Annu. Rev. Cell Biol. 6:539-557 (1990)), the residues do not appear to be important for specificity. We conclude that van der Waals packing differences do not have a dominant role in the discrimination between the Fos-Jun heterodimer and the corresponding homodimers. Rather, the mechanism of specific heterodimer formation appears to be predominantly electrostatic in nature.

This conclusion is supported further by the finding that the $N_{e,g}$ hybrids show pH-dependent stability similar to that observed with the native peptides. In particular, the Fos $N_{e,g}$ homodimer exhibits very strong pH-dependent stability ($T_m = 41^\circ\text{C}$ at pH 7 and $>90^\circ\text{C}$ at pH 4). In contrast, the pH dependence of the $N_{a,d}$ hybrid dimers does not resemble that of the native peptides. Thus, residues at positions e and g in the Fos and Jun sequences also account in large part for the dramatic pH-dependent stabilities observed with the native peptides.

Discussion

The requirements for specificity in the Fos-Jun system appear to be simple: 8 residues from Fos and from Jun, in a background of the GCN4 leucine zipper, are sufficient to mediate preferential heterodimer formation. pH dependence studies suggest a mechanism for specificity in which destabilization of the Fos homodimer by acidic

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residues (at positions e and g) shifts the dimerization equilibrium toward the Fos-Jun heterodimer. Therefore, preferential heterodimer formation by the Fos and Jun leucine zipper peptides is largely a thermodynamic consequence of Fos homodimer instability (O'Shea, E.K. et al., Science 245:646-648 (1989)). Destabilization of a homodimer is also used to provide specificity in the case of the tropomyosin $\alpha\beta$ heterodimer (Lehrer, S.S. et al., Science 246:926928 (1989); Lehrer, S.S. and W.F. Stafford, III, Biochemistry 30:5682-5688 (1991)).

The coupling of the ionization state of residues at positions e and g to the stability of the Fos and Jun leucine zippers can be rationalized by using the crystal structure of a peptide corresponding to the GCN4 leucine zipper (O'Shea, E.K. et al., Science 254:539-544 (1991)). In this crystal structure, the methylene groups of the predominantly charged residues at positions e and g pack against the predominantly hydrophobic residues at positions a and d. Thus, the hydrophobic interface is actually formed by side chains from 4 residues of the heptad repeat. Additionally, terminal charged groups of residues at positions e and g of the preceding heptad are close to each other. It is likely that the close proximity of negatively charged residues at positions e and g of opposing Fos monomers would disrupt the complementary packing seen at the dimer interface of the coiled coil, accounting for the instability of the Fos homodimer at neutral pH.

Example 2 Synthesis of Peptide ACID-p1 and Peptide BASE-p1

Peptides were synthesized using small-scale FMOC HBTU reaction cycles and acetic anhydride capping on an Applied Biosystems Model 431A peptide synthesizer (for a review, see S.B.H. Kent, Annu. Rev. Biochem. 57, 957 (1988)). Peptides were cleaved from the resin with trifluoroacetic

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acid (TFA) using standard cleavage methods and were de-salted on a Sephadex G-10 column in 5% acetic acid. Final purification was by reverse-phase high performance liquid chromatography (HPLC) with a Vydac preparative C18 column (2.2 x 25 cm) at 25°C. A linear acetonitrile-H₂O gradient with segments of 0.1% to 0.2% buffer B increase per minute was used with a flow rate of 10 or 20 mL/min. Buffer A consisted of water with 0.1% TFA and buffer B consisted of 90% acetonitrile, 10% water with 0.1% TFA. Peptides were >90-95% pure, as judged by analytical HPLC. The identity of each peptide was confirmed by mass spectrometry on a Finnegan Lasermat and was found to be within 2 daltons of the expected mass.

Example 3 Characterization of Homodimers (ACID-p1/ACID-p1 and BASE-p1/BASE-p1) and Heterodimers (ACID-p1/BASE-p1)

CD studies were performed with a 1 mm, 0.5 cm or 1 mm cuvette on an Aviv Model 60DS or 62DS CD spectrophotometer equipped with a thermoelectric controller. All CD studies were done in the presence of phosphate-buffered saline (PBS: 150mM NaCl, 10 mM Na phosphate, pH 7.0). All peptide concentrations were determined by absorbance at 280 nm in 6 M GuHCl (26). Thermal melting curves were determined by monitoring the CD signal at 222 nm as a function of temperature. Melting temperatures were estimated by taking the first derivative of the CD signal with respect to temperature¹ (temperature in K) and finding the minimum of this function (35). Reversibility was checked for all thermal melts and, in general, melting curves performed at pH <8 are reversible (>90% recovery of starting CD signal).

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The ACID-p1/BASE-p1 Heterodimer is Helical and Stable

Circular dichroism (CD) spectra of ACID-p1 and BASE-p1 at 37°C in phosphate-buffered saline (PBS), pH7 indicate that the ACID-p1 and BASE-p1 peptides are predominantly unfolded). In contrast, the characteristic the helical minima at 222 and 208 nm in the spectrum of an equimolar mixture of ACID-p1 and BASE-p1 indicate that the mixture is highly helical. At 0°C, a 100 μ M mixture of ACID-p1 and BASE-p1 is ~ 100% helical ($[\theta]_{222} = -34,000$). The helical structure formed by the ACID-p1 and BASE-p1 mixture is stable, as it undergoes a cooperative unfolding transition when denatured with urea. In contrast, the isolated peptides show no evidence for cooperative unfolding. Sedimentation equilibrium studies of ACID-p1 and BASE-p1 at 20°C in PBS, pH 7 indicate that this mixture is heterodimeric. Thus, the ACID-p1 and BASE-p1 peptides associate preferentially and fold as a stable, helical heterodimer.

The amide proton exchange behavior of the ACID-p1 and BASE-p1 heterodimer was characterized by nuclear magnetic resonance (NMR). One-dimensional NMR spectra were recorded on a Bruker AMX 500 MHz spectrometer at 20°C with a sweep width of 6024.1 Hz and a recycle time of 1.2 sec. 8192 points were collected and water was suppressed by continuous irradiation. Trimethylsilylpropionic acid was used as a standard (A. DeMarco, J. Magn. Reson. 26, 527 (1977)). Data were processed with the FTNMR software package (provided by Dr. Dennis Hare, Hare Research, Inc.) and spectra were apodized with a gaussian window. Since the global stability of the ACID-p1 and BASE-p1 heterodimer under the exchange conditions is -6.7 kcal/mol, the expected maximum degree of protection from exchange is $\sim 10^5$. At a time when protons that are protected by a factor of $\sim 10^3$ are expected to be half exchanged, many proton resonances have full intensity. Additionally, at a

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later time point corresponding to a protection of 10^4 , twelve amide proton resonances are present in the spectrum. Therefore, the ACID-pl and BASE-pl heterodimer has slowly-exchanging amide protons with protection factors near those expected from the global stability of the heterodimer, suggesting that the molecule has a well-packed 3^0 interface.

Characterization of the Disulfide-Bonded Heterodimer and Homodimers

Our design predicts that the ACID and BASE peptides will fold as parallel, helical dimers, but at reasonable peptide concentrations the isolated ACID-pl and BASE-pl are too unstable to study. One way to stabilize dimeric coiled-coil peptides is to join the peptides with a flexible disulfide bond. Therefore, versions of the ACID and BASE peptides (ACID-plN and BASE-plN) containing an N-terminal cysteine followed by two glycines were synthesized (glycines were added to allow disulfide bond formation without the distortion of the coiled-coil structure.) CD studies demonstrate that the disulfide-bonded heterodimer and homodimers are $> 80\%$ helical at 0°C and that each dimer shows a cooperative thermal unfolding transition indicating that the ACID and BASE peptides can be stabilized sufficiently with a disulfide bond to permit folding as stable, helical dimers.

The concentration dependence of stability was studied for each of the disulfide-bonded peptide dimers to determine if the orientation of the helices is parallel and if the dimers associate to a higher-order oligomers. When the ACID-plN and BASE-plN heterodimer and homodimer are joined in the parallel orientation, the stability of each dimer is independent of peptide concentration over a range of concentration from $\sim 2.5 \mu\text{M}$ to $170 \mu\text{M}$. In contrast, a heterodimer disulfide-bonded in the antiparallel orientation

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ion has a CD signal that is dependent on peptide concentration, indicating that the antiparallel dimers are associating to higher-order oligomers. The antiparallel heterodimer consists of the peptide ACID-plN (N-terminal Cys-Gly-Gly) disulfide bonded to a BASE peptide that has a C-terminal sequence of Gly-Gly-Cys. In PBS containing 2.25 M GuHCl at 0°C, a 0.9 μM sample of the antiparallel dimer has a $[\theta]_{222}$ value of -11,080 and a 24.5 μM sample has a $[\theta]_{222}$ value of -25,300. In contrast, the helicity of the parallel, disulfide-bonded ACID and BASE heterodimer and homodimers is independent of peptide concentration over a sample concentration range from -2.5 μM to 170 μM . Collectively, these experiments indicate that the ACID and BASE peptides are parallel and dimeric.

15 Mechanism of Specificity

Our design strategy sought to drive preferential heterodimer formation by destabilizing the homodimers rather than by stabilizing the heterodimer. If electrostatic destabilization of the homodimers is occurring, the homodimers will be unstable in conditions of neutral pH and low ionic strength and will become more stable when the charges are titrated to extremes of pH or when the ionic strength is increased. In contrast, if stabilization of the heterodimer by ion pairs is present, the heterodimer will be most stable at neutral pH and will become less stable as the charge on residues involved in ionic interactions is titrated at pH extremes. Thus, a bell-shaped pH dependence of stability curve would be observed. Additionally, if electrostatic stabilization is important, the heterodimer will be most stable at low ionic strength and will become less stable as residues involved in ionic pairs are shielded by ions.

The increased stability of the disulfide-bonded species allowed us to investigate the pH and ionic

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strength dependence of stability of the homodimers. The stability of both disulfide-bonded homodimers is strongly pH and ionic strength dependent. The ACID-p1N homodimer is > 80% more stable at acidic pH than at pH 7, demonstrating clearly that it is destabilized by acidic residues at neutral pH. Similarly, the BASE-p1N homodimer is destabilized at neutral pH by positively charged basic residues, as it becomes ~20°C more stable as the pH is changed from neutral to basic pH. Additionally, the disulfide-bonded homodimers are stabilized by increasing ionic strength. These results indicate that the homodimers are destabilized by unfavorable electrostatic interactions.

Interestingly, at neutral pH the stability of the BASE-p1N homodimer is much greater than that of the ACID-p1N homodimer. It is likely that part of the reason for the greater stability at neutral pH of the BASE-p1N homodimer as compared to the ACID-p1N homodimer is the difference in the length of the sidechains of residues at positions e and g. Whereas ACID-p1N has glutamate (containing two methylene groups) at position e and g, BASE-p1N has lysine (four methylene groups). The longer lysine sidechain allows for more flexibility and solvation of the terminal charged group. The idea that the length of sidechains at positions e and g is important in determining homodimer stability is supported by studies of BASE peptides containing the non-natural amino acids ornithine (three methylene groups) or diaminobutyric acid (two methylene groups). BASE homodimers containing these non-natural amino acids are predominantly unfolded and the stability of the corresponding ACID/BASE heterodimer decreases with the decreasing sidechain length. Two analogues of the BASE-p1N peptide were studied; one containing diaminobutyric acid at positions e and g (DAB-p1N), and the other containing ornithine at these positions

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(ORN-plN). Although the disulfide-bonded BASE-plN homodimer (containing lysine at positions e and g) has a T_m of 66°C, neither the disulfide-bonded DAB-plN homodimer nor the ORN-plN homodimer shows evidence for structure at 0°C or evidence for a cooperative thermal unfolding transition. Additionally, the disulfide-bonded heterodimers formed between ACID-plN and DAB-plN or ORN-plN have T_m 's of -63°C and -80°C, respectively (the ACID-plN/BASE-plN heterodimer has a T_m > 100°C).

10 In contrast to the stability of the homodimers, the stability of the heterodimer is relatively independent of ionic strength and pH. Because the disulfide-bonded ACID-plN/BASE-plN heterodimer cannot be thermally denatured in the absence of chemical denaturant, the pH and ionic strength dependence of the ACID-pl + BASE-pl mixture was 15 studied in the absence of a disulfide bond. From pH 4 to pH 9 the stability of the ACID-pl + BASE-pl heterodimer is relatively independent of pH. It is difficult to assess the stability of the heterodimer at pH extremes (3.5 > pH 20 > 10.5) because the ACID-pl + BASE-pl thermal denaturation curve contains more than one transition, suggesting that homodimers are populated significantly. It is puzzling that the heterodimer does not exhibit pH and ionic strength dependent stability, given that the heterodimer 25 has charged sidechains positioned to be able to form salt bridges (of the same type seen in the GCN4 leucine zipper structure). One possible explanation for the lack of pH and ionic strength dependent stability is that salt bridges form in the heterodimer but they are not significantly 30 stabilizing. There is precedent for this explanation, as the GCN4 leucine zipper peptides show little pH and ionic strength dependent stability even though salt bridges are seen in the crystal structure. Alternatively, the stabilizing effect of salt bridges could be coincidentally 35 offset by destabilizing intrahelical repulsion. It is

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plausible that such compensating effects exist, as the Fos/Jun heterodimer is more stable at low pH than neutral pH. The greater stability at low pH suggests that there is substantial intrahelical repulsion at neutral pH. In any case, these studies of pH and ionic strength dependence suggest that destabilization of the homodimers makes a major contribution to preferential heterodimer formation.

Quantitation of Specificity

Because the preference for heterodimer in the ACID and BASE peptides is so large, the ratio of heterodimer to homodimer cannot be measured readily from an equilibrium mixture of the two peptides. However, the degree of specificity can be estimated because K_{spec} (the equilibrium constant describing the ratio of heterodimer to homodimer) is linked thermodynamically to the dissociation constants for each of the dimers. Therefore, the dissociation constants for each dimer were determined so that the degree of specificity, $\Delta G_{\text{spec}} (= -RT \ln K_{\text{spec}})$, could be estimated. The following quantitation of specificity should be considered an estimate because the linkage relationship relating the dissociation constants relies upon the assumption that the monomer-dimer equilibria are two-state.

The dissociation constant for the ACID-p1N and BASE-p1N heterodimer was determined by monitoring the CD signal as a function of urea concentration. These resulting denaturation curve was fit to a two-state model for monomer-dimer equilibrium to obtain a dissociation constant for the heterodimer of 3×10^{-8} M. A two-state model for unfolding was assumed and the urea denaturation curve for the heterodimer was fit with a non-linear least squares fitting program (KaleidaGraph, Synergy Software) to the following equation that describes the GuHCl dependence of the CD signal for a monomer-dimer equilibrium: $\theta = \theta_d +$

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$$(\theta_m - \theta_d) [-e^{(-\Delta G^* + m[G_{\text{GNC}}])/RT}/4C_T + ((e^{(-\Delta G^* + m[G_{\text{GNC}}])/RT})^2/16C_T^2 + e^{(-\Delta G^* + m[G_{\text{GNC}}])/RT}/2C_T)^{1/2}],$$

where θ = CD signal at 222 nm; θ_d = guanidine dependence of the CD signal of the dimer; θ_m = guanidine dependence of the CD of the monomer; ΔG^* = free energy of folding under standard state conditions of 20°C and 1 M concentration of peptide chains; m = slope of the guanidine dependence of ΔG ; R = gas constant; T = temperature in K; C_T = total concentration of peptide chains. From this fit, a dissociation constant of 3×10^{-8} was obtained. An estimate of the dissociation constant for each of the homodimers was obtained from measurements of the helical CD signal as a function of peptide concentration. The dissociation constant for the BASE-pl homodimer is $\sim 1 \times 10^{-3}$ M and that for the ACID-pl homodimer is $> 5 \times 10^{-3}$ M. These dissociation constants place a lower limit on the degree of preference for heterodimer, ΔG_{spec} , of -6.5 kcal/mol ($> 10^5$ -fold preference for heterodimer).

Specificity can also be estimated by measuring the difference between the melting temperature (T_m) of the heterodimer and the average of the T_m s (melting temperature) for the homodimers (ΔT_m). ΔT_m has been measured for other disulfide-bonded leucine zipper peptides and has been shown to be related to ΔG_{spec} by a proportionality constant of 7.4°C/kcal mol⁻¹. ΔT_m for the disulfide-bonded ACID-plN and BASE-plN peptides is $> 56^\circ\text{C}$. If the same proportional relationship between ΔT_m and ΔG_{spec} exists with the ACID and BASE peptides, this lower limit for ΔT_m implies that ΔG_{spec} is at least -7.5 kcal/mol ($> 10^{5.6}$ -fold preference for the heterodimer).

Thus, the heterodimer is preferred over the ACID-pl and BASE-pl homodimers by at least $\sim 10^5$ -fold. This degree of specificity is much greater than that observed for the Fos and Jun peptides where the Fos/Jun heterodimer is preferred by only $\sim 10^2$ -fold.

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Equivalents

Those skilled in the art will recognize, or be able to ascertain using not more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims.

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CLAIMS

1. A synthetic peptide consisting essentially of 30 amino acid residues, wherein the amino acid residues are as follows:
 - 5 a) amino acid residues 4, 11, 18, 25, 6, 13, 20 and 27 are negatively charged amino acid residues;
 - b) amino acid residues 7, 14, 21, 28, 3, 10, 17 and 24 are hydrophobic amino acid residues;
 - 10 c) amino acid residues 1, 2, 8, 15, 22, 29, 9, 16, 23 and 30 are small, uncharged amino acid residues; and
 - d) amino acid residues 5, 12, 19 and 26 are charged amino acid residues.
2. The synthetic peptide of Claim 1 wherein:
 - 15 a) the amino acid residues of (a) are glutamate residues;
 - b) the amino acid residues of (b) are leucine residues or aspartate residues;
 - 20 c) the amino acid residues of (c) are alanine residues or glutamine residues; and
 - d) the amino acid residues of (d) are glutamate residues, lysine residues or tryptophan residues.

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3. A synthetic peptide consisting essentially of 30 amino acid residues, wherein the amino acid residues are as follows:

- a) amino acid residues 4, 11, 18, 25, 6, 13, 20 and 27 are positively charged amino acid residues;
- b) amino acid residues 7, 14, 21, 28, 3, 10, 17 and 24 are hydrophobic amino acid residues;
- c) amino acid residues 1, 2, 8, 15, 22, 29, 9, 16, 23 and 30 are small, uncharged amino acid residues; and
- d) amino acid residues 5, 12, 19 and 26 are charged amino acid residues.

4. The synthetic peptide of Claim 3 wherein:

- a) the amino acid residues of (a) are lysine residues;
- b) the amino acid residues of (b) are leucine residues or aspartate residues;
- c) the amino acid residues of (c) are alanine residues or glutamine residues; and
- d) the amino acid residues of (d) are glutamate residues, lysine residues or tryptophan residues.

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5. A helical heterodimer consisting essentially of two synthetic peptides, designated peptide ACID-p1 and peptide BASE-p1, wherein:

- 5 a) peptide A consists essentially of 30 amino acid residues, wherein the amino acid residues are as follows:
- (1) amino acid residues 4, 11, 18, 25, 6, 13, 20 and 27 are negatively charged amino acid residues;
- 10 (2) amino acid residues 7, 14, 21, 28, 3, 10, 17 and 24 are hydrophobic amino acid residues;
- (3) amino acid residues 1, 2, 8, 15, 22, 29, 9, 16, 23 and 30 are small, uncharged amino acid residues; and
- 15 (4) amino acid residues 5, 12, 19 and 26 are charged amino acid residues
- b) peptide B consists essentially of 30 amino acid residues, wherein the amino acid residues are as follows:
- 20 (1) amino acid residues 4, 11, 18, 25, 6, 13, 20 and 27 are positively charged amino acid residues;
- (2) amino acid residues 7, 14, 21, 28, 3, 10, 17 and 24 are hydrophobic amino acid residues;
- 25 (3) amino acid residues 1, 2, 8, 15, 22, 29, 9, 16, 23 and 30 are small, uncharged amino acid residues; and
- 30 (4) amino acid residues 5, 12, 19 and 26 are charged amino acid residues.

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6. The helical heterodimer of Claim 5 wherein:

a) in peptide ACID-p1:

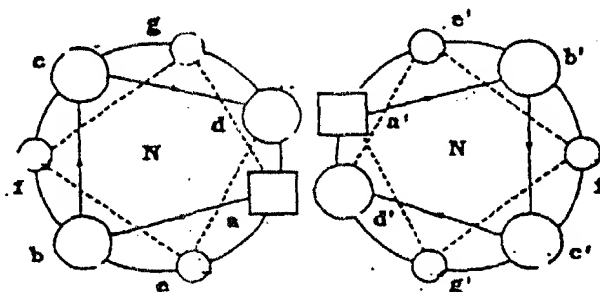
- (1) the amino acid residues of (a) are glutamate residues;
- 5 (2) the amino acid residues of (b) are leucine residues or aspartate residues;
- (3) the amino acid residues of (c) are alanine residues or glutamine residues; and
- 10 (4) the amino acid residues of (d) are glutamate residues, lysine residues or tryptophan residues.

b) in peptide BASE-p1:

- (1) the amino acid residues of (a) are lysine residues;
- 15 (2) the amino acid residues of (b) are leucine residues or aspartate residues;
- (3) the amino acid residues of (c) are alanine residues or glutamine residues; and
- 20 (4) the amino acid residues of (d) are glutamate residues, lysine residues of tryptophan residues.

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7. A coiled-coil heterodimer comprising at least one pair of synthetic peptides, represented by the following helical wheel:



- wherein the first member of the peptide pair is designated peptide ACID-p1 and the second member of the peptide pair is designated peptide BASE-p1 and:
- a) the amino acid residues at positions e and g of peptide A are negatively charged amino acid residues;
 - b) the amino acid residues at positions e' and g' of peptide B are positively charged amino acid residues;
 - c) the amino acid residues at positions a and d of peptide A and positions a' and d' of peptide B are hydrophobic amino acid residues;
 - d) the amino acid residues at positions b and c of peptide A and positions b' and c' of peptide B are uncharged amino acid residues; and
 - e) the amino acid residues at position f of peptide A and position f' of peptide B are charged amino acid residues.

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8. The coiled-coil helical heterodimer of Claim 7 where-
in:
- a) the amino acid residues of (a) are glutamate residues;
 - 5 b) the amino acid residues of (b) are lysine resi-
dues;
 - c) the amino acid residues of (c) are leucine resi-
dues;
 - 10 d) the amino acid residues of (d) are alanine resi-
dues or glutamine residues; and
 - e) the amino acid residues of (e) are glutamate
residues or lysine residues.
9. A multi-unit peptide comprising at least two synthe-
tic peptides of Claim 1.
- 15 10. A multi-unit peptide comprising at least two synthe-
tic peptides of Claim 3.
11. A coiled-coil heterodimer of Claim 7, further com-
prising more than one pair of synthetic peptides
ACID-p1 and BASE-p1.
- 20 12. A chimeric peptide comprising a synthetic peptide of
Claim 1 and a second peptide which is not a synthetic
peptide of Claim 1.
13. A chimeric peptide of Claim 12, wherein the second
component is selected from the group consisting of:
25 peptides, polypeptides, glycopeptides, small organic
molecules and detectable labels.
14. A chimeric peptide of Claim 13, which is detectably
labeled.

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15. A multi-unit peptide comprising at least two chimeric synthetic peptides, each chimeric synthetic peptide comprising at least one synthetic peptide of Claim 1 and a second component which is not a synthetic peptide of Claim 1.
5
16. A multi-unit peptide of Claim 15, wherein the second component is selected from the group consisting of: peptides, polypeptides, glycopeptides, small organic molecules and detectable labels.
- 10 17. A multi-unit peptide of Claim 16 which is detectably labeled.

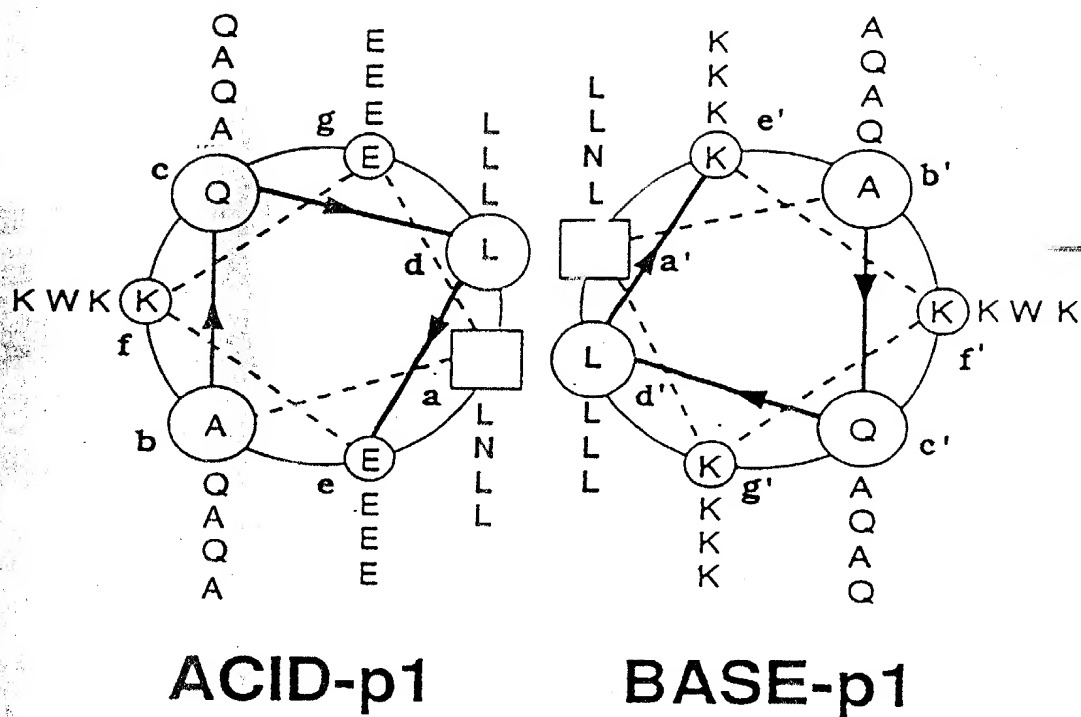


FIGURE 1

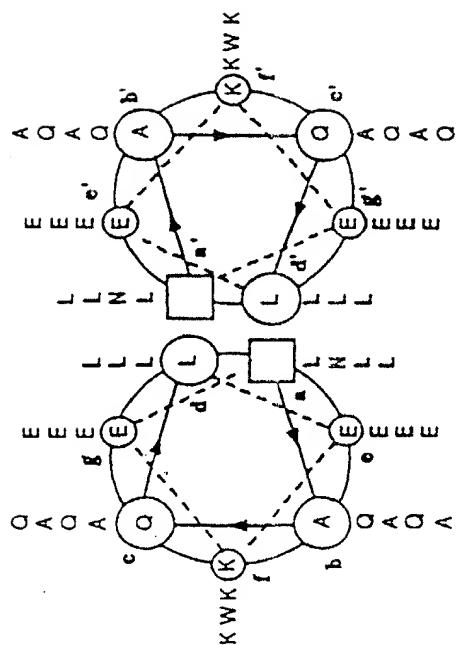


FIGURE 2A

ACID-p1

ACID-p1

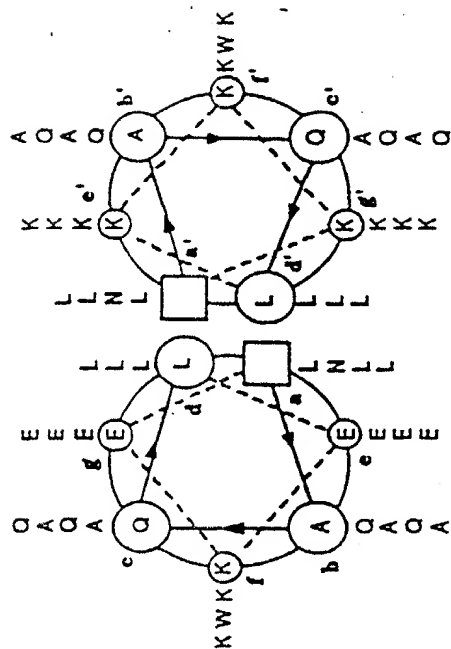


FIGURE 2B

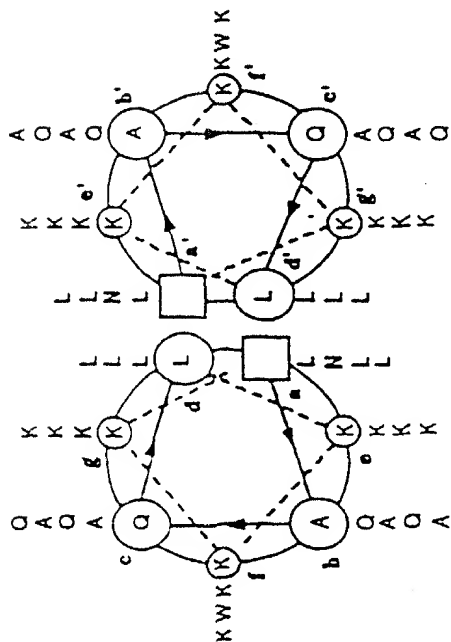


FIGURE 2C

BASE-p1

ACID-p1

BASE-p1

BASE-p1

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FIGURE 3

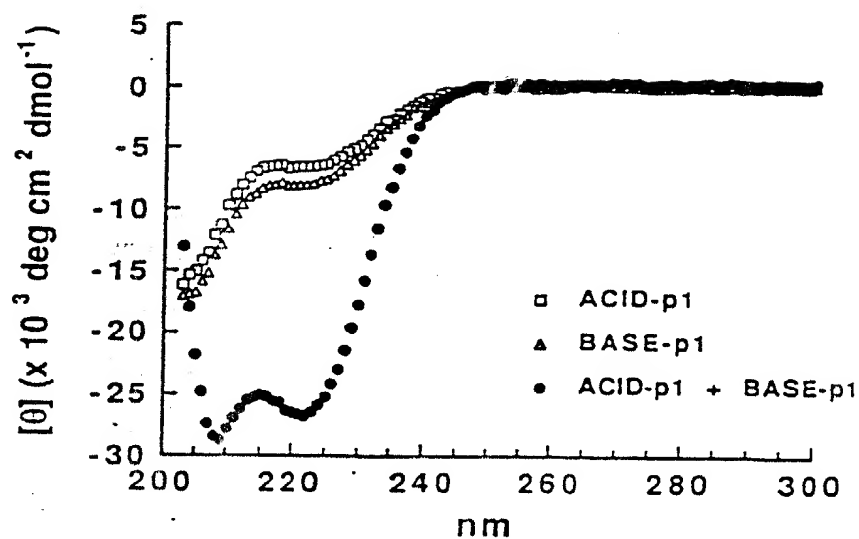
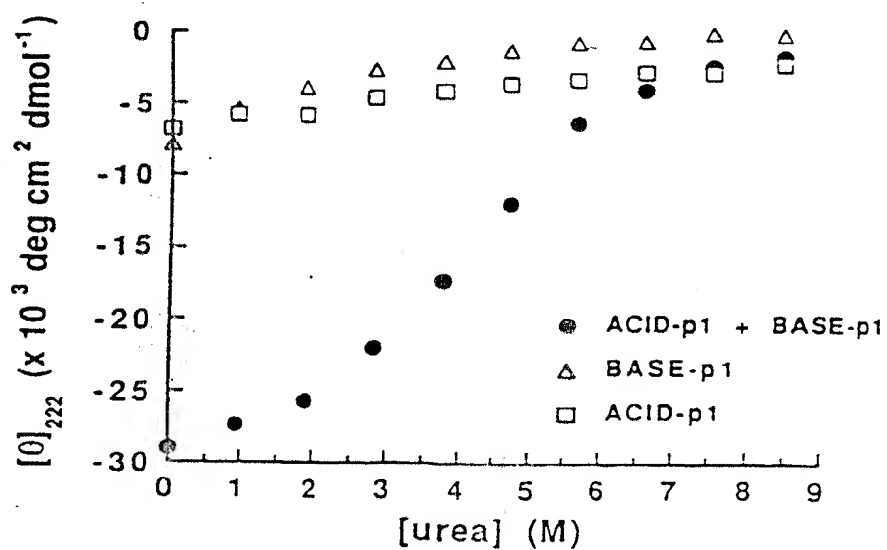


FIGURE 4



SUPPLEMENTARY SHEET

INTERNATIONAL SEARCH REPORT

International Application

PCT/US 93/00884

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)*	
According to International Patent Classification (IPC) or to both National Classification and IPC	
Int.Cl. 5 C07K7/10	
II. FIELDS SEARCHED	
Minimum Documentation Searched ⁷	
Classification System	Classification Symbols
Int.Cl. 5	C07K
Documentation Searched other than Minimum Documentation to the extent that such Documents are Included in the Fields Searched ⁸	

III. DOCUMENTS CONSIDERED TO BE RELEVANT ⁹		
Category ⁶	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³
A	JOURNAL OF BIOLOGICAL CHEMISTRY. (MICROFILMS) vol. 256, no. 3, 10 February 1981, BALTIMORE, MD US pages 1214 - 1224 R. S. HODGES ET AL. 'Synthetic Model for Two-stranded alpha-Helical Coiled-coils' cited in the application see part of 'Results and Discussion' on pages 1215-1218 see figure 1; table 1 --- -/--	1-11
<p>⁶ Special categories of cited documents: ¹⁰</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"A" document member of the same patent family</p>		
IV. CERTIFICATION		
Date of the Actual Completion of the International Search		Date of Mailing of this International Search Report
15 JUNE 1993		28 -06- 1993
International Searching Authority		Signature of Authorized Officer
EUROPEAN PATENT OFFICE		FUHR C.K.B.

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
A	JOURNAL OF BIOLOGICAL CHEMISTRY. (MICROFILMS) vol. 259, no. 21, 10 November 1984, BALTIMORE, MD US pages 13253 - 13261 S.Y.M. LAU ET AL. 'Synthesis of a Model Protein of Defined Secondary and Quaternary Structure' cited in the application see page 13259, right column, paragraph 2 - page 13260, right column, paragraph 2; figure 2; table 1 ---	1-11
P,A	WO,A,9 210 567 (CREATIVE BIOMOLECULES, INC.) 25 June 1992 see page 13, paragraph 1 - page 14, paragraph 1; claims 1-15 -----	1-15

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Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A-9210567	25-06-92	US-A- 5197973 AU-A- 9132391	30-03-93 08-07-92